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14. ABSTRACT
Purpose: Investigate the delivery of camptothecin in the presence of vitamin D3 with triblock copolymer-derived nanospheres that will increase the solubility of both of the drugs and provide protection to the camptothecin's lactone ring, resulting in its increased bioavailability to breast cancer cells. To date we have done the evaluation of two nanosphere formulations containing short alkyl pendent chain (DTB-SA/5K, Butyl) and/or benzyl ring (DTBn-SA/5K) Drug-binding efficiency of these nanospheres was evaluated for a constant quantity of the nanospheres with varying concentrations of the CPT and/or VD3. HPLC methods were developed and validated for quantitative determination of CPT and VD3 in the copolymer systems. To elucidate the synergistic effect of vitamin D3 on increasing the binding efficiency of CPT, different CPT to VD3 feed ratio were investigated. Results: The binding efficiency of VD3 in the presence of CPT. VD3 binding efficiency is not affected by the presence of the camptothecin, but is strongly affected by the nanospheres composition. Work was delayed by contract difficulties at Rutgers, but is now underway.

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To whom it might concern:

I would like to bring to your attention that although this research project was awarded in August 2006 the actual award document was not issued until after April 2007. As a consequence, the actual research has begun just 3 month ago. We also applied for a one-year no-cost extension of funding that was accepted only on August 13, 2007. Thus, the below report is a summary of preliminary data obtained within 3.5 month of a research work.

Introduction

Background

Self-assembling nanospheres offer a promising route to the delivery of pharmaceuticals that have poor bioavailability by improving the drugs' stability, circulation times in the body, and permeability through cell membranes, while reducing their toxicities.(*1*) Many drugs, including anti-tumor agents, anti-depressants and statins, are lipophilic and therefore require a solubilization process to enable their parenteral delivery.(*2*) Of the many alternative approaches proposed to overcome the obstacle of poor bioavailability of the drug, perhaps the most promising is the use of amphiphilic block copolymers that self-assemble into supramolecular nanoparticles.(*3*) These nanoparticles can be designed to provide stable dispersions of hydrophobic drugs with low cytotoxicity, thus making them attractive alternatives to less mechanically stable liposomes or more cytotoxic surfactant dispersant systems such as the CremophorEL which has been associated with some of Taxol's serious clinical side effects.(*4*) The amphiphilic block copolymers typically form a core – shell architecture in which the hydrophobic core serves as the reservoir for the incorporation of liphophilic drugs and diagnostic agents(*5*) and the hydrophilic shell enables stable dispersion in an aqueous environment and frequently also offers protection from protein adsorption and subsequent biological attack.(*6*) Amphiphilic block copolymers with poly(ethylene glycol)(*7*) as the hydrophilic block and polyester,(*8, 9*) poly(amino acid),(*10-12*) or polyether(*13, 14*) as the hydrophobic block have been explored for applications in drug delivery. Particle size has been shown to be a critical design parameter, as particles with diameters less than 200 nm and having PEG shells avoid entrapment by the reticuloendothelial system (RES) and accumulate preferentially in tumors that typically exhibit an enhanced permeability and retention effect (EPR).(*5, 15*) The biodistribution and uptake by the tumor of the nanoparticle is further dictated by charge density, conformation, hydrophobicity, and immunogenicity.(*16, 17*) The drug loading efficiency of the nanoparticles is also governed by a number of critical parameters, particularly the chemical affinity of the loaded drug for the nanoparticle core.(*18-20*)

We have recently reported on the design and synthesis of an unique ABA-type amphiphilic triblock copolymers that self-assemble into nanospheres at low critical aggregation concentration.(*21-23*) The A-blocks of these copolymers are composed of poly(ethylene glycol) PEG and the B-blocks are composed of polyarylate oligomers of desaminotyrosyl-tyrosine alkyl esters (DTR) and non-toxic diacids (Scheme 1). The FDA has recently approved one of the polyarylate formulations for use in a hernia repair medical device. Tyrosine-derived triblock copolymers self-assemble into spherical structures with hydrodynamic diameters between 50 nm and 100 nm, thus providing particle size and surface chemical properties superior to conventional drug delivery designs.(*21-23*) In addition to their biocompatibility, biodegradability and lack of cellular toxicity, these nanospheres strongly bind and retain *in vitro* anti-tumor cytotoxicity of the hydrophobic chemotherapeutic agent, paclitaxel.(*21-23*) *In vivo* efficacy of nanosphere-paclitaxel formulation exhibited anti-tumor activity in a breast cancer xenograft model that is similar to that of an equivalent dose of clinically used formulation of Cremophor-paclitaxel.(*23, 24*) It is our believe, that this novel technology can potentially address the key military and civilian requirements for effective breast cancer chemotherapy: nontoxic administration, increased bioavailability, prolonged circulation and targeting cancer cells, leading to substantially greater drug efficacy and lower toxicity. Further exploration of the proposed multidisciplinary research, while potentially high risk, may result in the introduction of innovative, high impact treatments for breast cancer.

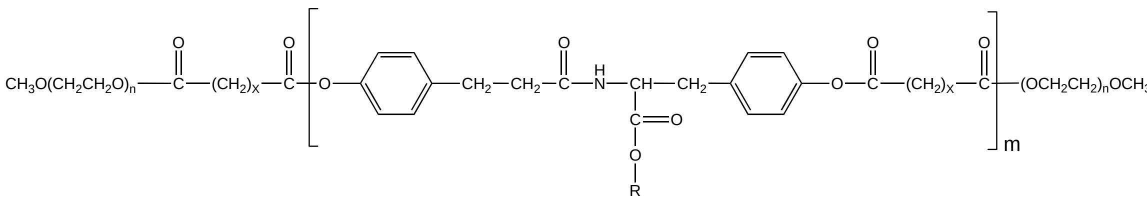
Rationale Camptothecin and its derivatives, such as 9-aminocamtothecin and 9-nitrocamptothecin, are inhibitors of topoisomerase I and have been investigated for their chemotherapeutic activity and inhibition of human breast carcinoma cells.(*25*) The integrity of the lactone ring system of camptothecins is a key determinant for the chemotherapeutic efficacy. The hydrolytic instability of camptothecins and their hydrophobic nature have complicated clinical development of these compounds.(*26*) It is postulated that our nanosphere delivery of this class of drugs will be far superior to other available methods and will open new avenues for adjuvant therapies such as simultaneous administration of several hydrophobic anticancer drugs with different mechanism of activity. Thus, complexation of vitamin D3, another hydrophobic chemotherapeutic and chemopreventive agent,(*27*) with our nanospheres in the presence of 9-nitrocamptothecin may provide a novel pathway in breast cancer treatment.

Objectives It is been proposed to investigate multifunctional targeted nanospheres that may be capable of overcoming the physicochemical and biological barriers to breast cancer drug delivery. Our goal is to parenterally deliver multiple therapeutic agents at high local concentrations and with physiologically appropriate timing directly to cancer cells, thereby interrupting the growth and metastasis of the tumor. Our initial focus will be on the delivery of 9-nitrocamptothecin with triblock copolymer-derived nanospheres that will increase the solubility of the drug and provide protection to the lactone ring, resulting in increased bioavailability to breast cancer cells. In order to decrease toxicity to normal cells, we will design and validate tumor-specific, targeted nanosphere drug complexes. Moreover, we will evaluate the relative efficacy and potential synergies of nanospheres containing 9-nitrocamptothecin alone, vitamin D3 alone, mixtures of these complexes delivered simultaneously, and a single nanosphere complex containing both drugs.

Body

Special Note on Nomenclature The abbreviation, DTR-XA/5K, is used to designate the various copolymer compositions in the family of ABA triblocks copolymers. The PEG A-blocks are abbreviated as 5K, indicating the molecular weight and units of the PEG components (i.e., 5K = PEG5000). The oligo B-blocks are distinguished by both their alkyl pendent chain "R" linked to the DTR (desaminotyrosyl-tyrosine alkyl ester) unit and/or the diacid "XA" to form the DTR-ester (DTR-XA). The three pendent chains "R" used are (B) butyl, (O) n-octyl, (D) n-dodecyl or (Bn) benzyl and the diacid "XA" is (SA) suberic acid (Scheme 1). Therefore, DTO-SA/5K stands for the triblock copolymer PEG_{5K}-*b*-oligo(desaminotyrosyl-tyrosine octyl ester suberate)-*b*-PEG_{5K}. Additional abbreviations are VD3 for vitamin D3 and CPT for camptothecin.

Copolymer Syntheses and Nanosphere Formulation The first objective of this study will involve determination of triblock copolymer structure-activity relations (SAR's) for optimum binding of camptothecin and vitamin D3 and the evaluation of process improvements so as to achieve the highest possible stable nanosphere-drug complex concentrations in aqueous solutions. Systematic synthetic variations were made in the copolymer structures (Scheme 1) to expand the range of nanosphere hydrophobicities. The synthesis of desaminotyrosyl-tyrosine esters, DTR,*(28)* and triblock copolymers has been previously described.*(22, 23, 29)* Briefly, the triblock copolymers were synthesized in a one-pot reaction at 20 °C using *in situ* carbodiimide coupling of the PEG and oligo(DTR-XA) while reaction conditions such as monomer ratios, temperature and reaction time were kept constant for all compositions.



R = DTB (Butyl, C4); DTO (Octyl, C8); DTD (Dodecyl, C12), DTBn (Benzyl, C7)
X = SA, Suberate (C6)

Scheme 1. Structure of PEG-*b*-oligo(DTR-XA)-*b*-PEG triblocks copolymers

We expect to identify an optimum ratio of triblock hydrophobicity/hydrophilicity, determined by the physical and chemical properties of the copolymer blocks, that provides for effective delivery of each selected drug. To this end the following triblock copolymer compositions were synthesized and characterized.

Table 1. Molecular weight properties of the PEG_{5K}-*b*-oligo(DTR-XA)-*b*-PEG_{5K} triblock copolymers and their corresponding nanospheres hydrodynamic diameters

Copolymer/nanospheres composition	Mn	Mw	Mw/Mn	DP[a]	Nanospheres hydrodynamic diameter, nm[b]
DTB-SA/5K	20000	27000	1.35	18	69 ± 1.5
DTO-SA/5K	21000	29000	1.36	18	*55 ± 1.3*
DTD-SA/5K	24000	32000	1.33	21	72 ± 1.6
DTBn-SA/5K	22500	29600	1.31	21	76 ± 1.7

[a] DP, degree of polymerization, was determined by the following equation: $(Mn_{DTR\text{-}XA/5K} - 2x(Mn_{mPEG}))/MW_{DTR\text{-}XA}$
[b] Cumulant fit. The SD value was for the nanosphere mean hydrodynamic diameter obtained for the three measurements of a single batch.

The chemical structure of the tyrosine-based triblock copolymers with varying pendent R chains is illustrated in Scheme 1. With a copolymer synthesis reaction time of one hour, the copolymers are obtained with narrow molecular weight distributions centered on 29 kDA (Table 1). Based on the copolymers investigated so far, it can be concluded that the copolymer molecular weights are not strongly affected by the pendent ester in the DTR monomers.
The triblock copolymers were induced to self-assemble in dilute aqueous solution using a conventional injection method.(*29*) The resulting turbid dispersion was sequentially

filtered through 0.45, 0.22 and 0.1 micrometer size syringe filters and the final filtrate was used for all subsequent characterizations. The triblock copolymer nanospheres have hydrodynamic diameters that go through a minimum size as the pendent ester chain lengths increase from ethyl (C4) to octyl (C8) to dodecyl (C12) (Table 1). This apparent minimum is reminiscent of the Ferguson effect observed in surfactant systems.(*30*) Given that the B-block chain lengths, as reflected by their degree of polymerization (DP) are very similar (DP ~ 19), it can be suggested that the DTO-containing nanospheres will have the most densely packed hydrophobic cores. The degree of polymerization was also measured by ^{1}H NMR and very similar values were obtained (data not shown). The observed variations in self-organization behavior as a function of the DTR-XA core-forming blocks are consistent with their thermal properties.(*31, 32*) Poly(DTB-XA)'s are semi-amorphous materials characterized by a glass transition and they can be readily plasticized by water. In contrast, poly(DTD-XA)'s possess long range structural order with highly layered mesogenic properties, while poly(DTO-XA)'s have less ordered structures typical in non-mesogenic macromolecules. An increase in the length of the core-forming block is expected to cause an increase in the core size of the nanospheres which, in turn, may result in an increased drug loading capacity per nanosphere.(*33*) In conclusion, all of the copolymer formulations and their resultant nanospheres investigated so far appear to be suitable for use in drug delivery based on their structural composition, polymer molecular weight distribution and nanosphere size.

Nanospheres Drugs Compatibility and Binding Efficiency

Our initial focus was on the delivery of camptothecin in the presence of vitamin D3 with triblock copolymer-derived nanospheres that will increase the solubility of both of the drugs and provide protection to the camptothecin's lactone ring, resulting in its increased bioavailability to breast cancer cells. To this end, we began with evaluation of two nanosphere formulations containing short alkyl pendent chain (DTB-SA/5K, Butyl) and/or benzyl ring (DTBn-SA/5K). The rationale for choosing these two formulations is based on different packing densities of the resultant nanospheres. With shorter R groups, there can be more flexible packing while the introduction of the benzyl group might affect the rigidity and therefore self-assembly organization of the nanospheres. The presence of π-π interaction between the aromatic group and/or double bond of drug molecules and the phenyl group of DTBn pendent chain could increase the loading efficiency and stability of nanospheres-drug complexes.

Drug-binding efficiency of these nanospheres was evaluated for a constant quantity of the nanospheres with varying concentrations of the CPT and/or VD3. HPLC methods were developed and validated for quantitative determination of CPT and VD3 in the copolymer systems. To elucidate the synergistic effect of vitamin D3 on increasing the binding efficiency of CPT, different CPT to VD3 feed ratio were investigated.

Figure 1 represents the binding efficiency of VD3 in the presence of CPT. VD3 binding efficiency is not affected by the presence of the camptothecin, but is strongly affected by the nanospheres composition. Measured binding efficiency of VD3 by DTB-containing nanospheres was 60% while DTBn-containing nanospheres retained only 46% of the drug. Interestingly, the binding efficiency of nanospheres made of a mixture of DTB and DTBn-containing polymers (1:1 wt/wt) was similar to the one of DTB-containing nanospheres suggesting, at first, that less rigid nanospheres core organization is needed to maximize the encapsulation of VD3. It should be noted that the drug-binding efficiency was measured following meticulous purification process, which includes filtration through

0.22 μm filters, ultracentrifugation and additional filtration through 0.22 μm filters for sterilization purposes. The initial filtration step strongly effects the drug binding efficiency because all nanosphere-drug particles and particles alone that are larger then 220 nm will be removed. It was found that this filtration step reduced nanosphere yield as well as drug content in the nanospheres by 25% for VD3-containing nanospheres and 35% in camptothecin-bounded nanospheres.

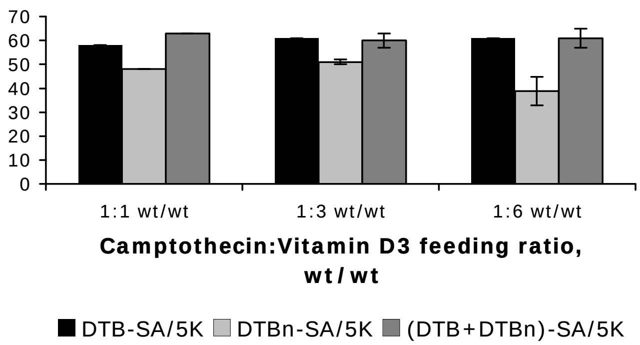


Figure 1. Vitamin D3 binding efficiency as function of camptothecin concentration and composition of the core-forming oligomers. Data expressed as ± SD of three independent measurements.

Figure 2 depicts the binding efficiency of CPT as a function of VD3 concentration and nanospheres' composition. Different trend was observed in camptothecin binding: CPT binding efficiency is strongly affected by the presence and concentration of Vitamin D3.

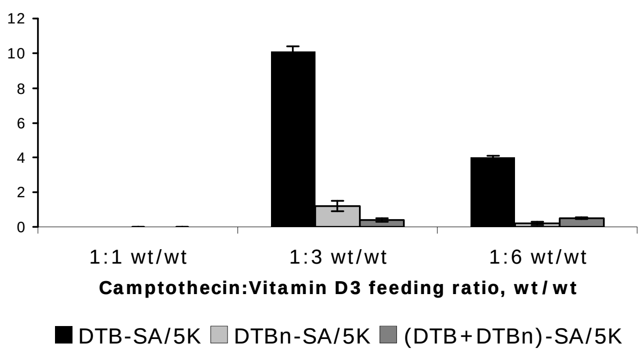


Figure 2. Camptothecin binding efficiency as function of vitamin D3 concentration and composition of the core-forming oligomers. Data expressed as ± SD of three independent measurements.

Binding efficiency increases with increasing CPT to VD3 feed ratios between 1 to 3 (wt/wt) and then decreases at higher feed ratios (Figure 2). Also nanospheres composition has a strong effect on the CPT binding: the presence of a shorter and more flexible pendent chain caused more CPT to be retained by the nanospheres. This suggests that the presence of π-π interaction plays an impairing role during encapsulation and later stability of CPT-loaded nanospheres. However, we would still suggest that there is a strong complexation between the DTBn-containing nanospheres and CPT. This hypothesis can be explained by the difficulties in re-suspending drug-loaded DTBn-containing nanospheres during purification process. We refer to purified nanospheres as those that were processed as follows: the self-assembled drug-loaded nanosphere suspensions were filtered through 0.22 μm filters; the filtered drug-loaded nanospheres were isolated by ultracentrifugation at 65 000 rpm (290 000 x g) for 3 h at 25 °C, followed by removal of the supernatant; the pelleted drug-loaded nanospheres were then washed twice with PBS and re-suspended with gentle agitation in 1 mL of PBS at 25 °C. Finally, the re-suspended pellets were again filter-sterilized (0.22 μm).

Therefore, at this point we cannot definitely conclude if the presence of benzyl group as a pendent chain decreases nanospheres encapsulation of the CPT and subsequently VD3 (see Figure 1) or low biding efficiency of both of the drugs should be attributed to the low recovery of drug/s-loaded nanospheres after purification. As a consequence, in the future studies we will use the ultra-filtration as an alternative methodology for drug-loaded nanospheres purification.
An important observation is that at the optimum conditions of drugs feed ratio and nanospheres composition, CPT's binding efficiency is still 5 times lower then VD3. This can be explained by unique physical properties of CPT: despite its high log D value (oil:water partition coefficient), it is still poorly soluble in most organic solvents and has the tendency to self-aggregate. This explanation is supported by visual observations of a heavy yellow precipitates of CPT at all feed ratio's and for all nanosphere compositions. The precipitation was observed during preparation and purification processes. In contrast, VD3-conatining nanospheres showed good stability within the nanospheres at all complex formations and purification steps suggesting that vitamin D3 has greater compatibility with tyrosine-derived nanospheres.

Further investigation and optimization of tyrosine-derived nanospheres as a novel pathway in breast cancer treatment are ongoing and to be reported in the completion of the research program.

Key Research Accomplishments

1. Copolymer syntheses and nanosphere formulation
2. Preliminary studies of nanospheres and drugs compatibility

Reportable Outcomes

No reportable outcomes have yet to result from the research conducted in the last 4 month.

Conclusion

As stated in the beginning of this report, due to the delay in funds transferring the actual research has begun just a several month ago. Even thought we are confident that this work may result in the introduction of innovative treatments for breast cancer, at this point we do not have enough confirmation to comment or summarize the implications of the completed research.

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